Determination of Dicumarol Metabolites in Bile of Rats

WALTER D. CONWAY ˣ, FRANCIS H. LEE, and LAWRENCE NEUFELD

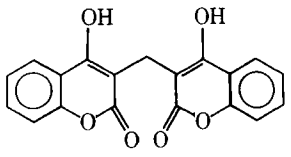
Abstract □ After intravenous administration of dicumarol-^{14}C to rats, the bile excreted over the next 24 hr contained from 32 to 46% of the administered radioactivity. At least three primary metabolites and a small amount of unchanged dicumarol were present in the bile. Over 91% of the primary metabolites was converted to dicumarol and 7-hydroxydicumarol by hydrolysis with β-glucuronidase. Some primary metabolites were hydrolyzed simply by acidification to pH 3 or by treatment under the acidic conditions utilized in the enzymatic hydrolysis. The three primary metabolites contain carboxylic acid groups, as indicated by their electrophoretic mobility–pH profiles, and some are simple glucuronides of dicumarol and 7-hydroxydicumarol. The possibility that others are derivatives of these compounds in which a coumarin lactone ring is opened cannot be ruled out. When the metabolites released by either acidification or enzymatic hydrolysis were chromatographed in *n*-butanol–3 *M* ammonia, artifacts were produced, presumably as a result of decomposition of 7-hydroxydicumarol. The question is raised whether a previously reported metabolite (B055) is an artifact.

Keyphrases □ Dicumarol and metabolites—biliary excretion after intravenous administration of dicumarol-^{14}C, rats □ Biliary excretion—dicumarol and metabolites after intravenous administration of dicumarol-^{14}C, rats

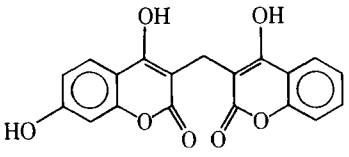
The anticoagulant drug dicumarol (I) was prepared in the early 1940's and is still utilized in therapy (1, 2). It is known to be almost totally metabolized in the body, and several investigators reported separation of from four to seven metabolites in the feces, urine, or bile of rats by paper chromatography (3–9). None of these metabolites has been identified, and it is not known whether any possesses physiological activity (10). While biliary excretion has been recognized as a major pathway for elimination of dicumarol, few studies have been made on bile (8, 9, 11, 12), and in only two of these (8, 9) were the metabolites separated.

The most extensive study (7) of the structure of a metabolite concerned a metabolite isolated from the feces of rats and designated as B055 to signify its mobility of 0.55 in the paper chromatographic system of *n*-butanol–3 *M* ammonia (1:1). This metabolite contained a hydroxy group in position 7 of one coumarin ring; it was neither a glucuronide nor identical with 7-hydroxydicumarol (II).

In the present study, the excretion of radioactivity in the bile, urine, and feces of rats was measured after intravenous administration of dicumarol. Metabolites were separated by high-voltage paper electrophoresis and by paper chromatography. The amounts of I and II were measured by the reverse isotope dilution technique in aliquots of 0–24-hr bile treated alternatively as follows: (*a*) with β-glucuronidase, (*b*) by incubation with buffer in the absence of enzyme, and (*c*) by chloroform extraction of unhydrolyzed bile.



I



II

EXPERIMENTAL

Dicumarol-4,4′-^{14}C (specific activity 0.74 μCi/mg) was synthesized by a modification of the method of Underwood (13) and was administered to one rat. Dicumarol-methylene-^{14}C (specific activity 11.8 μCi/mg) was prepared by the method of Lee *et al.* (14, 15) and administered to two rats. Only one radioactive spot was observed when electrophoresis and paper chromatography were carried out in the systems to be described. 7-Hydroxydicumarol was prepared by the method of Abramovitch and Gear (16) and was identical with an authentic sample[1].

Electrophoresis—Separations by high-voltage paper electrophoresis were run on Whatman 3 MM paper for 30 min at 5000 v at 20° using an enclosed apparatus with two cooling platens[2]. The electrophoregrams were dried and the radioactive zones were detected by scanning in a gas flow counter using methodology previously described (17). Scanner settings were 50 cm/hr, time constant 10 sec, and 5 × 50-mm open window.

Paper Chromatography—Bile samples were examined by applying aliquots of bile as a streak across the entire width of 4 × 40-cm strips of Whatman No. 1 (10-μl aliquot) and No. 3 MM (15-μl aliquot) papers, followed by descending development using *n*-butanol–3 *M* ammonia (1:1, v/v) (3–9). Aliquots (10 μl) of 0.2% solutions of dicumarol and 7-hydroxydicumarol in 0.1 *N* NaOH were applied to the same paper strips as reference compounds. The reference compounds were located by their fluorescence under short wavelength (254 nm) UV light. Radioactive zones were located by scanning in the same manner as for electrophoregrams.

Animal Treatment—Polyethylene 10 cannulas were secured with ligatures in the proximal and distal portions of the bile ducts of male Sprague–Dawley rats (~200 g) under ether anesthesia. The bile duct was then ligated in two places between the cannulas and severed between the ligatures. The cannulas were led through puncture wounds in the backs of the animals, and the exteriorized ends were connected with a 1.5-cm length of 27-gauge needle tubing, thereby permitting normal circulation of bile. The procedure is similar to that recently described by Rietbrock and Abshagen (18). Animals were housed in a restraining cage and given water and food *ad libitum*.

Twenty-four hours after surgery, the cannula was opened to permit collection of bile. Approximately 1 ml of a solution of dicumarol in 0.1 *N* NaOH was administered *via* the femoral vein. Samples of urine and bile were collected at 24-hr intervals. The bile collection vessel was immersed in ice throughout the collection period, and samples were counted and analyzed as soon as possible after collection. In one rat the 0–48-hr feces were collected on a screen.

Enzymatic Hydrolysis—Samples of bile were hydrolyzed with β-glucuronidase using the following microprocedure. A 100-μl aliquot of bile was diluted with water to 1 ml, and 100 mg of monobasic potassium phosphate was added followed by approximately 1 mg of the appropriate nonradioactive carrier (either dicumarol or 7-hydroxydicumarol) in approximately 75 μl of 0.2 *N* NaOH. The

[1] The gift of 7-hydroxydicumarol from Dr. R. A. Abramovitch is gratefully acknowledged.

[2] Shandon model 2550.

Table I—Elimination of Radioactivity in Bile, Urine, and Feces after Intravenous Administration of Dicumarol-^{14}C to Rats

Rat Number	Dose, mg/kg	Hours Postmedication	Percent of Administered ^{14}C Eliminated in			
			Bile	Urine	Feces	Total
7	24[a]	0–24	45.8	21.8	Not measured	67.6
		24–48	6.5	3.1	—	9.6
		0–48	52.3	24.9	—	77.2
9	24[b]	0–24	42.6	20.6	—	63.2
		24–48	7.8	2.7	—	10.5
		0–48	50.4	23.3	3.2	76.9
10	16[b]	0–24	31.8	18.2	Not measured	50.0
		24–48	5.5	8.4	—	13.9
		0–48	37.3	26.6	—	63.9

[a] Specific activity 0.74 μCi/mg. [b] Specific activity 11.8 μCi/mg.

sodium hydroxide was added in 5-μl increments under the control of a microglass electrode to a final pH of 5.4. Then 5 μl of 4 *M* acetate buffer (prepared to yield a solution of pH 5.4 when diluted 25-fold) containing 6.3 mg/ml of bovine serum albumin and 100 μl of a β-glucuronidase preparation[3] were added. The sulfatase contained in the β-glucuronidase preparation is inhibited by the phosphate incorporated into the mixture (19). The sample was incubated at 37° in a water bath for 24 hr. When larger quantities of bile were hydrolyzed, the volumes of reagents were increased proportionately.

Reverse Isotope Dilution—The sample of hydrolyzed bile was extracted with two 20-ml portions of chloroform, and a 100-μl aliquot of each extract was counted to determine the recovery of radioactivity. One hundred milligrams of the appropriate carrier (either dicumarol or 7-hydroxydicumarol) was added to the combined chloroform extracts, and the solution was evaporated to dryness. The residue was recrystallized four to six times from 20 ml of acetic acid. Samples of solid were removed after each recrystallization, dried, and dissolved in 1–2 ml of boiling acetic acid, which, in turn, was dissolved in a dioxane-based scintillation fluid for assay of radioactivity.

Total radioactivity associated with the added carrier was calculated from the mean count rate of samples from the last two recrystallizations after constant specific activity had been reached. Samples of untreated bile or bile incubated without enzyme were similarly treated. In some cases, aliquots of bile or hydrolyzed bile were simply evaporated *in vacuo*, carrier was added, and the residue was recrystallized from acetic acid.

Liquid Scintillation Counting—Total radioactivity in urine, bile, and other solutions was assayed by dissolution of aliquots in a scintillation fluid consisting of dioxane (946 ml), water (50 ml), 2,5-diphenyloxazole (9.5 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.3 g), and naphthalene (142 g) and counting at ambient temperature using a liquid scintillation spectrometer[4].

Feces were homogenized with an approximately equal weight of 2% polysorbate 80[5]; a weighed aliquot was dried and prepared for scintillation counting by a modification of the plastic bag combustion technique (20). Samples of urines and combusted feces were counted for 10 min, providing over 10,000 accumulated counts, and were corrected for efficiency by the internal standard technique using a 109,000-dpm aliquot of toluene-^{14}C counted for 1 min.

RESULTS AND DISCUSSION

Excretion of Radioactivity—In early studies in this laboratory using anesthetized rats, the excretion of radioactivity in bile appeared to be somewhat less than would be expected from the reported excretion in feces of normal, conscious rats (6). Therefore, in subsequent experiments, the drug was administered to conscious animals after recuperation from surgery.

To allow animals sufficient time to recover from surgery and at the same time prevent the depletion of bile salts, which would occur with a simple exteriorized cannula, two cannulas were installed, as described in the *Experimental* section. This system permitted normal circulation of bile during the 24-hr recuperation. The drug was then administered intravenously and bile, urine, and feces were collected from the conscious animals.

Substantial amounts of radioactivity were obtained in the 0–24- and 24–48-hr bile (Table I), although these quantities are still somewhat less than the mean excretion of 57.2 and 10.6% in the 0–24- and 24–48-hr feces, respectively, of three normal rats dosed intravenously by Christensen (6). The excretion observed in urine was quite comparable to his values of 17.7 and 3.0% for 0–24- and 24–48-hr periods, respectively. This finding suggests that biliary excretion slows down as bile constituents are depleted over the long collection period.

Analysis by Electrophoresis—Analysis of 0–24-hr bile samples by direct application to paper, without extraction, and electrophoresis at pH 6.25 (Fig. 1) demonstrates the presence of at least three major components along with minor amounts of 7-hydroxydicumarol and dicumarol. Similar results were obtained by electrophoresis at pH 3.70 and 8.10.

Portions of the pH–mobility profiles of these metabolites in the bile of Rat 9 are presented in Fig. 2. Similar results were obtained for Rats 7 and 10. Lack of mobility below pH 2.7 rules out the possibility of a sulfuric acid ester metabolite (17), which would be ionized at low pH and would migrate substantially. The pKa in the region of 3.2, indicated by the pH–mobility profiles of three metabolites, is characteristic of carboxylic acids. The profile of the slowest moving component, accounting for about 9% of the radioactivity, is consistent with the profile observed for dicumarol.

After hydrolysis of bile (Rat 10) with β-glucuronidase, direct electrophoretic analysis of the hydrolysate (Fig. 3) demonstrates the absence of the metabolites originally present and the appearance of incompletely resolved zones in the vicinity of reference spots of authentic dicumarol and 7-hydroxydicumarol. Lack of exact correspondence of the zones observed by scanning with the positions of the authentic reference compounds should not be regarded as evidence of nonidentity. Under the conditions used for the electrophoresis, in which the authentic reference compounds

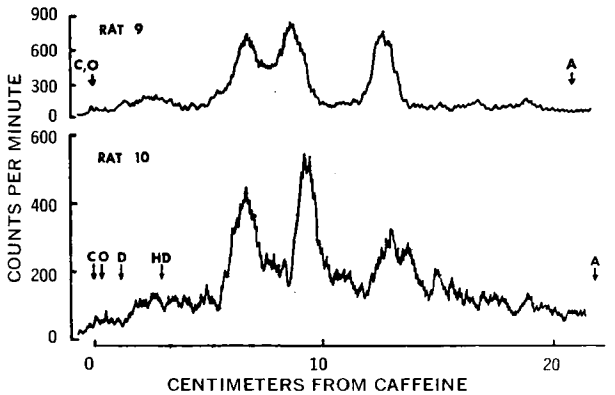


Figure 1—*Electrophoretic separation of metabolites in 10 μl of 0–24-hr biles of Rats 9 and 10, using phosphate buffer, pH 6.25, ionic strength 0.05. Positions of origin (O), caffeine (C), p-nitrobenzenesulfonic acid (A), dicumarol (D), and 7-hydroxydicumarol (HD) reference spots are indicated.*

[3] Glusulase, Endo Products, Inc., Richmond Hill, N.Y.
[4] Packard Tri-Carb model 2202.
[5] Tween 80.

Table II—Composition of 0–24-hr Bile as Measured by Reverse Isotope Dilution Analysis prior to and following Hydrolysis with β-Glucuronidase[a]

Rat Number	Percent of Radioactivity in Bile Present as: Dicumarol	7-Hydroxydicumarol	Total
After Treatment with β-Glucuronidase, 24 hr, 37°, pH 5.4			
7	50.8	40.7	91.5[b]
9	24.8, 23.8	69.7	94.5
10	39.9	55.2	95.1
After Incubation with Buffer Only, 24 hr, 37°, pH 5.4			
9	24.9	23.5	48.4
10	7.7	11.1	18.8
No Incubation with Buffer			
9	1.5	2.3	3.8[c]
After Evaporation of Bile and Recrystallization of Residue and Carrier from Acetic Acid			
7	11.6	21.4	33.0[b]

[a] One hundred microliters of bile was incubated with buffer or buffer plus β-glucuronidase and extracted with two 20-ml portions of chloroform; 100 mg of carrier was added to the chloroform, recovered, and recrystallized from acetic acid. [b] Four milliliters of bile was evaporated *in vacuo*, and 100 mg of carrier was added and recrystallized from acetic acid. [c] Not incubated with buffer but extracted and treated as in Footnote *a*.

were run in a channel adjacent to the bile on the electrophoregram, a discrepancy in relative mobility of the magnitude observed in Fig. 3 is commonly observed. The reference compound typically runs slightly faster than the corresponding compound in the biological fluid.

Analysis by Reverse Isotope Dilution—The composition of 0–24-hr bile samples, as determined by reverse isotope dilution analysis, is summarized in Table II. After hydrolysis with β-glucuronidase, 91.5–95.1% of the radioactivity in the 0–24-hr bile was accounted for as dicumarol and its 7-hydroxy derivative. Very little dicumarol or its 7-hydroxy derivative was present in the bile prior to subjecting it to the conditions for enzymatic hydrolysis. This finding is particularly evident from the results on the bile from Rat 9 where the bile was simply extracted with chloroform; 20% of the radioactivity in the bile was extractable into chloroform, but only a small portion of this amount, corresponding to 1.5 and 2.3% of the bile radioactivity, was characterized as dicumarol and 7-hydroxydicumarol, respectively, by reverse isotope dilution.

Either incubation with buffer alone or direct recrystallization of bile and carrier from acetic acid converts a significant portion of the nonhydroxylated metabolites to dicumarol, apparently all of them in the case of Rat 9. From 20 to 52% of the 7-hydroxylated metabolites are converted to 7-hydroxydicumarol by similar treatment.

If it is assumed that some conjugates are labile under acidic conditions, the data are consistent with the conclusion that the metabolites excreted in bile are predominantly simple glucuronides of

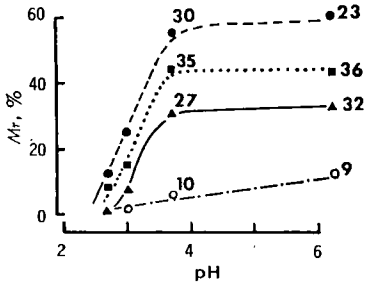


Figure 2—*Mobility* (M_r) *of metabolites in 0–24-hr bile of Rat 9 in the pH 2–6 region expressed as percent of mobility of* p-*nitrobenzenesulfonic acid. Numerals indicate percentage of bile radioactivity associated with the zone. Symbols* (○, ▲, ■, *and* ●) *represent the four zones observed.*

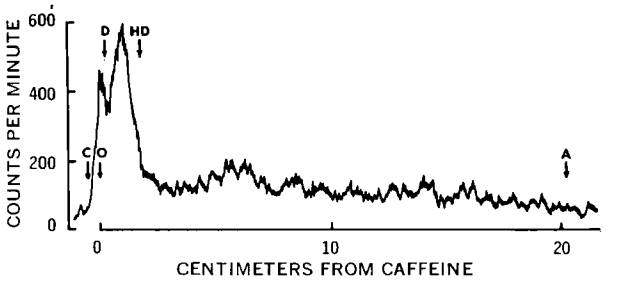


Figure 3—*Electrophoretic separation of components in the 0–24-hr bile of Rat 10 after hydrolysis with β-glucuronidase, using phosphate buffer, pH 6.25, ionic strength 0.05. Positions of origin (O), caffeine (C),* p-*nitrobenzenesulfonic acid (A), dicumarol (D), and 7-hydroxydicumarol (HD) reference spots are indicated.*

dicumarol and 7-hydroxydicumarol. At least 50% of the 7-hydroxylated metabolites, equivalent to at least 20% of the radioactivity in bile, appear to be simple glucuronides of 7-hydroxydicumarol. The nonhydroxylated metabolites may include a fairly labile glucuronide as well as other substances that can be converted to dicumarol under acidic conditions. Compounds similar to III, a metabolite of ethyl biscoumacetate, reported by Pulver and Kaulla (21) would behave in this manner.

Analysis by Paper Chromatography—Paper chromatography in *n*-butanol–3 *M* ammonia (1:1) has been utilized extensively in studies of the metabolic fate of dicumarol (3–9). The evidence presented here indicates that several artifacts are generated by chromatography of dicumarol metabolites in this system. The resulting confusion is no doubt responsible for the protracted period over which the disposition of dicumarol has been studied.

When bile is applied directly to paper and chromatographed in *n*-butanol–3 *M* ammonia (1:1), substantially all radioactivity remains near the origin (upper curve, Fig. 4). Little or no radioactivity is seen in the regions of control spots of dicumarol or 7-hydroxydicumarol or in the region expected for the reported metabolite B055. Acidification to pH 3 and extraction with ethyl acetate, as described by Buttar *et al.* (9), removed more than 95% of the radioactivity from bile. Chromatography of the ethyl acetate extract in the butanol–ammonia system revealed at least five components (lower curve, Fig. 4).

Similar chromatograms were obtained from the β-glucuronidase hydrolysate of 0–24-hr bile from Rat 10 by direct application of the hydrolysate to the paper (upper curve, Fig. 5) and from an extract (lower curve, Fig. 5) prepared by adjustment of the hydro-

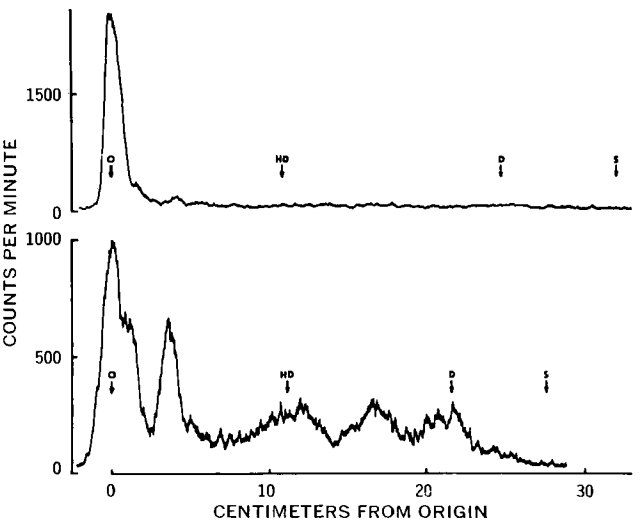
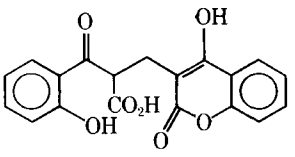


Figure 4—*Paper chromatography of 0–24-hr untreated bile from Rat 10 (upper curve) and ethyl acetate extract (lower curve) obtained from Rat 10 bile adjusted to pH 3 as described by Buttar* et al. *(9). Positions of origin (O), solvent front (S), and reference spots of dicumarol (D) and 7-hydroxydicumarol (HD) are indicated. The system is* n-*butanol–3* M *ammonia (1:1).*



III

lyzed bile to pH 3 and extraction with ethyl acetate (9).

The R_f values for the peaks and the percentages of bile radioactivity represented by the areas designated a, b, c, d, and e in the chromatograms shown in Fig. 5 are compared in Table III with the corresponding values obtained by Buttar *et al.* (9) by chromatography of an ethyl acetate extract of β-glucuronidase-hydrolyzed bile. The present results are in good agreement with those of Buttar *et al.* and confirm their conclusion that component e cochromatographs with dicumarol. The data presented here indicate that component c cochromatographs with 7-hydroxydicumarol.

The suggestion of the presence of at least five metabolites by the chromatographic data is at variance with the conclusion clearly indicated by reverse isotope dilution that virtually all radioactivity in bile is accounted for by dicumarol and its 7-hydroxylated derivative. This inconsistency arises only when the bile is hydrolyzed with enzyme (Fig. 4) or is simply acidified prior to extraction and chromatography (lower curve, Fig. 4) in the alkaline system. The additional components are not generated by chromatography of bile, which is neither acidified nor extracted (upper curve, Fig. 4). The reverse isotope dilution data (Table II) do suggest that some conjugates in bile are labile under acidic conditions.

Chromatography of dicumarol-^{14}C added to bile collected from an unmedicated rat chromatographed as a single zone, which corresponded to a reference spot of dicumarol in the *n*-butanol–3 *M* ammonia system. This finding eliminated the possibility of dicumarol decomposition or binding to biliary components to yield multiple spots.

Chromatography of nonradioactive 7-hydroxydicumarol produced two spots at R_f 0.38 and 0.65. The lower R_f spot was judged to be the unchanged 7-hydroxy compound by the similarity of the color produced on spraying with diazotized *p*-nitroaniline to that of the unchromatographed compound and also by the similarity of the R_f value to the value of 0.33 reported for it in this system by Christensen (7). After 3 days of standing in 3 *M* ammonia prior to chromatography, the spot at R_f 0.65 disappeared and at least five additional spots formed, ranging in R_f from 0.1 to 0.78. In contrast, nonradioactive dicumarol appeared to be stable after 3 days of standing in 3 *M* ammonia. Chromatography in the *n*-butanol–3 *M* ammonia system required at least 6 hours, which provided ample time for hydrolytic, oxidative, and possibly photochemical decomposition to occur.

Therefore, several metabolites observed by previous investigators apparently are artifacts resulting from the decomposition of 7-hydroxydicumarol by the widely used *n*-butanol–3 *M* ammonia solvent system. The 7-hydroxydicumarol can be released from some of its conjugates simply by acidification. The major artifact (Figs. 4 and 5) exhibits an R_f value in the range of the B055 metabolite (7). This raises the question of whether B055 is actually an artifact derived by decomposition of 7-hydroxydicumarol in the *n*-butanol–3 *M* ammonia solvent system in which the biological material was repeatedly chromatographed during the isolation procedure (7).

Table III—R_f Values and Relative Amounts of Radioactivity in Components Separated by Paper Chromatography in *n*-Butanol–3 *M* Ammonia (1:1) from the Enzyme-Hydrolyzed Bile of Rats Given Dicumarol-^{14}C Intravenously

	Upper Curve, Fig. 5[a]		Lower Curve, Fig. 5[b]		Buttar *et al.* (9)[c]	
Component	R_f	%	R_f	%	R_f	%
a	0.03	15.1	0.04	11.3	0.09	11.6
b	0.13	15.0	0.14	10.0	0.22	9.3
c	0.47	26.4	0.46	29.5	0.50	19.3
d	0.66	32.3	0.66	38.1	0.66	36.0
e	0.76	11.5	0.81	11.1	0.80	23.9
7-Hydroxydicumarol[d]	0.47	—	0.44	—	—	—
Dicumarol[d]	0.76	—	0.77	—	0.80	—

[a] β-Glucuronidase-treated 0–24-hr bile applied directly to chromatogram. [b] Ethyl acetate extract of acidified, β-glucuronidase-hydrolyzed 0–24-hr bile. [c] Ethyl acetate extract of acidified, β-glucuronidase-hydrolyzed 0–6-hr bile; mean values from Table 3 of Ref. 9. [d] Reference compounds run in adjacent channels on electrophoregram.

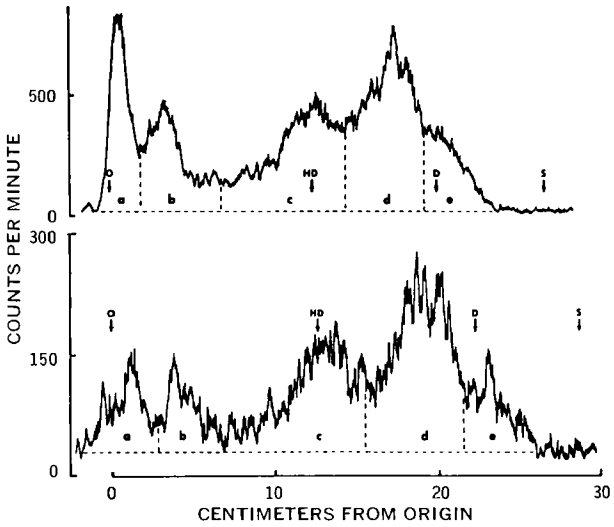


Figure 5—*Paper chromatography of β-glucuronidase hydrolysate (upper curve) of 0–24-hr bile from Rat 10 and paper chromatography of an ethyl acetate extract (lower curve) of the hydrolysate adjusted to pH 3 as described by Buttar* et al. *(9). Positions of origin (O), solvent front (S), and reference spots of dicumarol (D) and 7-hydroxydicumarol (HD) are indicated. The system is n-butanol–3* M *ammonia (1:1).*

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Correlations between Physical and Drug Release Characteristics of Polyethylene Glycol Suppositories

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Abstract □ The mechanical strength and elastic moduli of blocks of polyethylene glycol with a range of molecular weights were determined. A rotating-basket dissolution test was used to measure the release characteristics of prednisolone from similar blocks. The effects of blending bases of different molecular weight and of the addition of water also were determined. Linear relationships were found for the mechanical strength, molecular weight, and release rate, but no simple relationship could be observed for the elastic moduli.

Keyphrases □ Polyethylene glycol suppositories—relationships between physical characteristics (mechanical strength, molecular weight, and elastic moduli) and drug (prednisolone) release rates □ Suppositories, polyethylene glycol—relationships between physical characteristics and drug release rates □ Drug release characteristics of polyethylene glycols—relationship to polymer molecular weight, mechanical strength, and elastic moduli

Polyethylene glycols are among the most widely used of the hydrophilic polymer suppository bases. Drug liberation occurs as a result of base dissolution into the aqueous environment of the rectum, differing radically from the classical lipophilic bases which melt at body temperature and act as a lipid reservoir from which drug molecules partition prior to absorption.

Salicylate release from polyethylene glycol bases has been examined by several workers (1–5). High plasma levels of salicylic acid and sodium salicylate have been achieved (1) and the *in vitro* release of two different salicylates did not differ significantly (2). To achieve rapid drug release, polyethylene glycols have been recommended for aspirin formulations whereas cocoa butter (theobroma oil) is the base of choice for both sodium salicylate (4) and choline salicylate (3). The use of polyethylene glycols in some instances has produced similar plasma levels to an equivalent oral dose (4, 5). It has been suggested that salicylate liberation and absorption from polyethylene glycol suppositories may be influenced by salicylate–polyethylene glycol complexation (6–8).

Studies on drugs incorporated into a polyethylene glycol matrix include those on acetaminophen (9), iodoform and 2,4,6-triiodophenol (10), thiazinamium and indomethacin (11), chloramphenicol (12, 13), sulfonamides (14), antipyrine (phenazone) and sodium barbital (15), diphenhydramine and its hydrochloride salt (16), oxytetracycline (17), and other antibiotics (18). When selecting a suppository base, it is generally true that lipophilic drugs are best formulated in hydrophilic bases (19) and water-soluble compounds are best formulated in lipophilic bases (20) for rapid and complete release.

Rheological investigations of suppository bases have been confined to the molten or semisolid state (21–25) and determinations of "hardness" of the solid bases (4, 26–29). Baichwal and Lohit (25) ob-

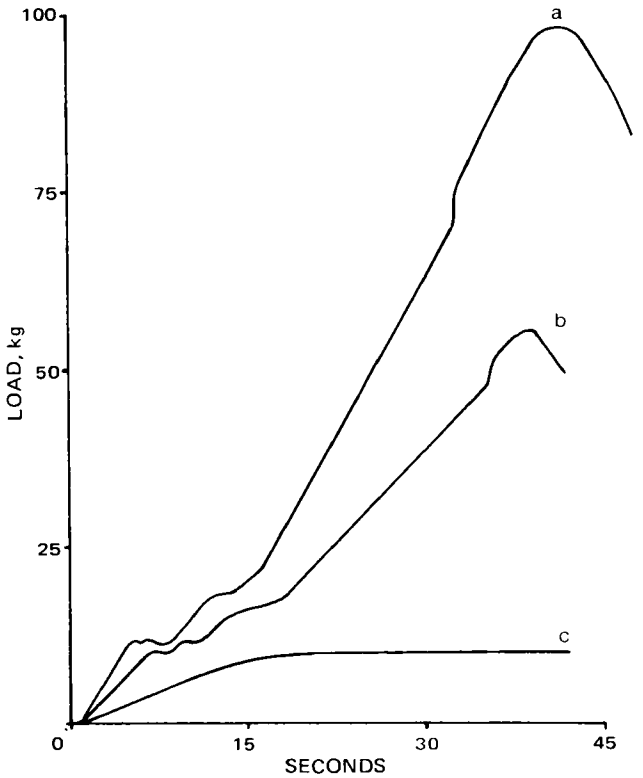

Figure 1—*Transmitted load-time curves for: (a) polyethylene glycol 6000, (b) 60% polyethylene glycol 6000 and 40% polyethylene glycol 1000, and (c) polyethylene glycol 1000. The rate of strain was 60 mm hr*$^{-1}$.